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## Purification and Characterization of an Extracellular 29-Kilodalton Phospholipase C from *Listeria monocytogenes*

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We purified and characterized an extracellular phospholipase produced by *Listeria monocytogenes*. This enzyme was separated as a homogeneous protein of 29 kDa by chromatography on DEAE-52 cellulose and Bio-Gel P100 columns. It is a zinc-dependent phospholipase C (PLC) that is mainly active at pH 6 to 7 and expresses lecithinase activity and a weaker sphingomyelinase activity. The exoenzyme also hydrolyzed phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin but not phosphatidylinositol. It was distinct from the 36-kDa phosphatidylinositol PLC produced by *L. monocytogenes* and from the *L. ivanovii* sphingomyelinase. The pure protein expressed a weak, calcium-independent hemolytic activity and was not toxic in mice. Western immunoblot analysis using a rabbit immune serum raised against the enzyme showed that all virulent strains of *L. monocytogenes* tested produced in the culture supernatant a 29-kDa PLC. In contrast, no proteins antigenically related to the 29-kDa PLC were detected in supernatants of *L. ivanovii*, *L. seeligeri*, *L. innocua*, or *L. welshimeri*. The role in virulence of the 29-kDa PLC specifically produced by *L. monocytogenes* remains to be established.

To our knowledge, the first major contribution of the virulence of *Listeria monocytogenes* derives from the pioneering work by G. B. Mackaness, demonstrating that virulent bacteria survive and even multiply within macrophages during the infectious process (24). Since then, *L. monocytogenes* has been extensively used by immunologists as a model to study the induction of T-cell-mediated immunity against intracellular parasites (3, 20, 35). Recent reports have established that a cytolytic toxin, listeriolysin O, is a major virulence factor promoting intracellular multiplication of *L. monocytogenes* (5, 8, 16, 28, 36) and even of the nonpathogenic species *Bacillus subtilis* (2). Although intracellular growth appears as a crucial event in the course of infection, expression of virulence involves several other critical steps, including penetration and invasion of intestinal mucosa, dissemination of bacteria in host tissues by cell-to-cell spreading (13, 32, 40) to create abscesses, and passage across the blood-brain barrier to cause meningoencephalitis. Most likely, these steps require several unknown virulence factors which are expressed in vivo during infection.

Among the putative virulence factors, the lipase activity found in culture supernatants of all *L. monocytogenes* strains must be considered. As first observed long ago (7, 38), strains of *L. monocytogenes* produce an extracellular lipolytic activity that induces a zone of opalescence around the colonies on egg yolk or lecithovitellin agar (11, 14, 15, 34, 39, 43). This lecithinase activity is distinct from listeriolysin O, having been recently identified as a 56-kDa thiol-activated cytolytic toxin devoid of any detectable enzymatic activity (9). It has been suggested that the lecithinase released in the culture supernatant of *L. monocytogenes* might be due to a phospholipase C (PLC) (21), but to our knowledge, this enzyme has not been identified or purified. In this work, we

purified and characterized the extracellular factor responsible for lecithinase activity. It is a zinc-dependent 29-kDa PLC that hydrolyzes lecithin and sphingomyelin but not phosphatidylinositol. This enzyme is weakly hemolytic and not toxic in mice. It is produced by all virulent strains of *L. monocytogenes* but has not been detected in related *Listeria* species.

### MATERIALS AND METHODS

**Bacterial strains and culture media.** For the purification of PLC, we used strain EGD (serovar 1/2a), originated from the Trudeau Institute (1982, Saranac Lake, N.Y.). The other bacterial strains used were *L. monocytogenes* N62262 (serovar 1/2a), N8018 (serovar 4b), N74217 (serovar 1/2b), N2661 (serovar 4b) (all wild strains from patients, from the Necker Hospital Collection, Paris, France), SLCC5156 (serovar 1/2a), SLCC3551 (serovar 4b), SLCC5132 (serovar 1/2a), and CIP82110<sup>T</sup> (serovar 1/2a); *L. ivanovii* CIP4141<sup>T</sup> (serovar 5); *L. seeligeri* CIP100100<sup>T</sup> (serovar 1/2); *L. innocua* CIP8011<sup>T</sup> (serovar 6); and *L. welshimeri* SLCC5334<sup>T</sup> (serovar 6) (CIP, Collection de l'Institut Pasteur; SLCC, Special *Listeria* Culture Collection, Würzburg, Germany).

For subcultures, brain heart infusion (BHI) broth or agar (bioMérieux, Marcy l'Etoile, France), tryptocasein soy agar (Diagnostics Pasteur, Marnes la Coquette, France), and 5% horse blood BHI agar were used. For production of the extracellular lecithinase, bacteria were grown on trypticase-glucose-yeast (TGY) broth prepared as follows: Biotrypticase (bioMérieux), 30 g; yeast extract (Difco Laboratories, Detroit, Mich.), 20 g; Na<sub>2</sub>HPO<sub>4</sub> · 12H<sub>2</sub>O, 8.3 g; KH<sub>2</sub>PO<sub>4</sub>, 0.7 g; and quartz-distilled water to 1,000 ml. This broth was supplemented with 0.1 mM ZnSO<sub>4</sub> and vegetable-activated charcoal (Prolabo, Paris, France) at 0.2% final concentration. In some experiments, ZnSO<sub>4</sub> was replaced by CuSO<sub>4</sub>, NiSO<sub>4</sub>, Co(NO<sub>3</sub>)<sub>2</sub>, or FeCl<sub>3</sub> (0.1 mM final concentration).

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After the pH was adjusted to 7.5, the culture broth was autoclaved at 115°C for 20 min. Sterile glucose was then added to a final concentration of 1% before inoculation. Lipolytic activity was detected on charcoal-treated TGY broth supplemented with 0.1 mM ZnSO<sub>4</sub> or BHI agar supplemented with 2.5% fresh egg yolk previously diluted in 0.15 M NaCl or in phosphate-buffered saline (PBS) (1:2, vol/vol). Colonies producing lipolytic activity were surrounded with a large opalescent zone revealing egg yolk degradation.

**Purification of PLC.** Bacteria were grown under shaking conditions for 9 h at 37°C in 2 liters of charcoal-treated TGY broth supplemented with 0.1 mM ZnSO<sub>4</sub> prepared as described above. After centrifugation at 10,000 × g for 15 min at 4°C, the supernatant fluid was 10-fold concentrated by rapid ultrafiltration at 4°C in an Amicon DC2 (hollow fiber H1 × 10:10-kDa cutoff). To purify the enzyme, we used a DEAE-52 (Whatman Biosystems, Maidstone, United Kingdom) column (40 by 2.6 cm) and Bio-Gel P100 (Bio-Rad Laboratories, Richmond, Calif.) columns (100 by 2.6 cm).

**Titration and characterization of PLC.** Phospholipase activity was titrated as described by Möllby et al. (30). Briefly, 100 µl of supernatant was incubated for 1 and 4 h at 37°C with 200 µl of lecithin (lecithin, 3.6 g [Merck Laboratories, Darmstadt, Germany]; sodium cholate, 2.4 g; ZnSO<sub>4</sub>, 1 mM final concentration; distilled water, 100 ml) and 700 µl of 0.15 M NaCl. Enzymatic activity was estimated turbidimetrically at 510 nm with an M25 spectrophotometer (Beckman, Brea, Calif.). One turbidimetric unit was arbitrarily defined as the amount of enzyme that causes an increase of absorbance of 0.100 within 1 h or, for very weak activity, 4 h. Protein concentration was determined by the method of Bradford (4) with Bio-Rad reagents. The isoelectric point of purified PLC was assessed in a pH gradient by isoelectrofocusing (Ampholin column type 8100; LKB Instruments Inc., Gaithersburg, Md.) according to the method of Haglund (12).

**Hemolytic assays.** The hemolytic activity of the purified PLC was estimated by incubating, for 18 h at 37°C in microtiter plates, 50 µl of serial twofold dilutions of the enzyme in 0.15 M NaCl (eventually supplemented with 1 mM CaCl<sub>2</sub>) with 50 µl of 2% erythrocyte suspensions (3 × 10<sup>8</sup> to 6 × 10<sup>8</sup> cells per ml) from various animal species (human, sheep, horse, guinea, pig, and rabbit). Hemolytic activity was expressed as the amount of pure PLC required to totally lyse 50 µl of 2% erythrocyte suspension after 18 h of incubation at 37°C.

**TLC.** The enzymatic specificity of the pure PLC preparation was analyzed by thin-layer chromatography (TLC) on Silica Gel 60 plates (Merck), using as substrates egg yolk lecithin, synthetic lecithin (L-α-phosphatidylcholine-dimyrystoyl), sphingomyelin, L-α-phosphatidylinositol, phosphatidylethanolamine, and phosphatidylserine (Sigma Chemical Co., St Louis, Mo.). We also used L-α-lysophosphatidylcholine (Sigma), obtained by the action of phospholipase A<sub>2</sub> on egg yolk, phosphorylcholine chloride, 1-monopalmitoylglycerol, and 1,2-dipalmitoylglycerol (Sigma) as controls. Briefly, 200 µl of substrate (36 mg/ml) dispersed in sodium cholate (24 mg/ml) diluted in 700 µl of 0.15 M NaCl supplemented with 100 µl of 10 mM ZnSO<sub>4</sub> was incubated for 1 and 4 h at 37°C with 5 µl of pure enzyme (75 µg/ml ~ 2,000 U/ml). In some experiments, EDTA (1 to 2 mM final concentration) was added to the reaction mixture. Then the phospholipids were extracted with 1 ml of chloroform-methanol (9:1, vol/vol) and evaporated under vacuum. Samples dissolved in 20 µl of chloroform were loaded onto silica gel plates. Ascending chromatography was carried out for 2 h in

chloroform-methanol-water (65:25:4, vol/vol/vol) or in hexane-diethylether-acetic acid (70:30:1, vol/vol/vol) to detect the release of diacylglycerol. Plates were then stained with iodine vapors or with ammonium molybdate to visualize the PO<sub>4</sub> components (29). Pure phospholipase C (alpha-toxin) from *Clostridium perfringens*, kindly provided by Colette Jolivet-Reynaud (Institut Pasteur, Paris), was used as a control.

The optimum pH of enzymatic activity was determined as described above by incubating for 1 h at 37°C, 5 µl of purified *L. monocytogenes* PLC (75 µg/ml ~ 2,000 U/ml) with 200 µl of lecithin dispersed in sodium cholate diluted in 800 µl of 0.15 M NaCl containing 1 mM ZnSO<sub>4</sub> adjusted at various pHs ranging from 5.0 to 8.5. Related phospholipids were then analyzed by ascending chromatography in chloroform-methanol-water and iodine staining as described above.

**Liposomes.** Liposomes were prepared by sequentially mixing 154 mg of phosphatidylcholine (Merck), 68 mg of cholesterol (Sigma), 25 ml of CHCl<sub>3</sub>, and 5 ml of methanol. After evaporation of the organic solvents at 31°C, lipids were dissolved in 3 ml of diethyl ether (Prolabo) and then added with 15 ml of distilled water. The mixture was then ultrasonicated for 4 min at 4°C and emulsified at 3,000 rpm/min with an Ultra-Turrax T25 (BioBlock Scientific, Illkirch, France). The molar ratio (phosphatidylcholine/cholesterol) was 7:4. Fifty microliters of liposomes was diluted in 940 µl of 0.15 M NaCl at various pHs (5 to 8.5) and incubated with 10 µl of purified enzyme (2,000 U/ml) for 4 h at 37°C. The A<sub>510</sub> was then measured with a Beckman M25 spectrophotometer.

**Amino acid composition and sequencing.** Sequence analysis was carried out with a gas-phase sequencer (model 470A; Applied Biosystems, Foster City, Calif.) equipped with an on-line phenylthiohydantoin amino acid derivative analyzer (model 120A) and run with the sequencing program recommended by the manufacturer as described previously (1). The pure 29-kDa PLC was electrotransferred on Immobilon (Millipore, Bedford, Mass.) and hydrolyzed by trypsin (Sigma). The peptides were purified by reverse-phase high-pressure liquid chromatography (HPLC) using a 0.1% trifluoroacetic acid-acetonitrile gradient and analyzed by a diode array detector (model 100S; Applied Biosystems). One apparently homogeneous peptide was further sequenced (1).

**SDS-PAGE and Western immunoblot analysis.** Proteins from the fractions obtained at various steps of the purification procedure were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Samples of 50 µl (5 to 10 µg of protein) were boiled for 2 min in 2% (wt/vol) SDS-5% (vol/vol) 2-mercaptoethanol-10% (vol/vol) sucrose-0.002% (wt/vol) bromophenol blue in 0.1 M Tris hydrochloride buffer (pH 6.8). Electrophoresis was performed in 7.5 to 20% (wt/vol) gradient polyacrylamide-0.1% SDS gels in 0.07 M Tris buffer (pH 8.8) for 18 h at 6 mA. The proteins were stained by Coomassie blue or silver nitrate.

For Western blot analysis, supernatants from bacterial strains grown for 9 h at 37°C in charcoal-treated TGY broth were passed through a 0.22-µm-pore-size filter and precipitated with 10% trichloroacetic acid. The precipitate was centrifuged at 10,000 × g at 4°C for 30 min and dissolved in 0.12 M Tris buffer (pH 6.8). Samples were boiled for 2 min as described above. Electrophoresis was performed in 13% (wt/vol) polyacrylamide-0.1% SDS in 0.37 M Tris buffer (pH 8.8) for 2 h at 150 mA. Proteins were electrophoretically transferred (1 h, 50 mA) onto nitrocellulose sheets (PH 79; Schleicher & Schuell, Dasse, Germany), in 0.2 M glycine-20% isopropanol 0.025 M Tris buffer (pH 8.3). The sheets were incubated for 1 h at room temperature with shaking in

PBS–10% Tween supplemented with 5% (wt/vol) skimmed milk (Regilait; France-Lait, Saint Marin-Belle Roche, France) prior to 1 h of incubation with rabbit anti-PLC diluted 1:2,000 in the buffer described above and then washed eight times in buffer before addition of 20 ml of milk buffer containing a peroxidase-labeled goat anti-rabbit immunoglobulin (Organon-Teknika-Cappel, Malvern, Pa.) diluted 1:1,000. Shaking was continued for 1 h at room temperature, and then the filters were washed six times in buffer supplemented with 0.1% Triton X-100. Enzymatic activity was revealed by addition of diaminobenzidine tetrahydrochloride (Sigma) supplemented with 0.02% hydrogen peroxide.

**Rabbit anti-29-kDa PLC serum and immunoabsorbed antibodies.** Female albino rabbits (3 kg) supplied by IFFA-Credo (Saint Germain sur l'Arbresle, France) were immunized by injecting subcutaneously 25 µg of purified PLC from *L. monocytogenes* emulsified in complete Freund's adjuvant on days 0, 7, and 14 and in incomplete Freund's adjuvant on day 90. Blood was collected before immunization and 2 weeks after the last injection. This antiserum was immunoabsorbed on purified 29-kDa PLC as follows. The pure 29-kDa PLC (75 µg/ml) was incubated on nitrocellulose sheets (BA85; Schleicher & Schuell) for 30 min at 22°C. After adsorption, the sheets were saturated with 5% skimmed milk as described above and then rinsed in PBS (pH 7.2). Rabbit anti-29-kDa PLC serum diluted 1:10 (PBS, pH 7.2) was incubated for 30 min at 22°C on nitrocellulose sheets in a volume of 5 ml. After extensive washings in PBS (pH 7.2), immunoabsorbed antibodies were eluted three times with 5 ml of glycine-HCl (pH 2.2) for 15 min at 22°C. Eluates were rapidly neutralized by addition of 1 M  $\text{PO}_4\text{HNa}_2$  and concentrated on a Centricon PM30 filter (Amicon) to a volume of 4 ml, corresponding to an optical density at 280 nm of 0.40. This immunoabsorbed anti-29-kDa PLC was then used for Western blot analysis.

**Toxicity in mice.** Specific-pathogen-free female ICR Swiss mice, 6 to 8 weeks old (Charles River, Saint Aubin les Elbeuf, France), were used. Groups of five mice were injected intravenously (i.v.) with increasing dose of purified PLC (1 to 25 µg per mouse), and mortality was observed over a period of 1 week.

## RESULTS

**Production of extracellular lecithinase in the culture supernatant of *L. monocytogenes*.** We first defined the optimal conditions to produce lecithinase activity in the culture supernatant from *L. monocytogenes*. Bacteria were grown at 37°C under shaking conditions, using TGY broth or charcoal-treated TGY broth supplemented or not with 0.1 mM  $\text{ZnSO}_4$ . Enzymatic activity was then titrated at successive times in the culture supernatant by a turbidimetric method, using lecithin as the substrate (30). Bacterial growth was identical in the four different culture conditions (Fig. 1). Enzymatic activity was undetectable when TGY broth was used and remained very weak when  $\text{ZnSO}_4$  was added to this broth. In contrast, enzymatic activity was significantly enhanced in charcoal-treated TGY broth, with a peak at 6 h (15 U/ml) and then a rapid decrease. When both charcoal and  $\text{ZnSO}_4$  were added to TGY broth, enzymatic activity reached 50 U/ml by 9 h, at a time when the stationary phase of bacterial multiplication was reached (Fig. 1). The lecithinase activity was not stimulated when  $\text{Zn}^{2+}$  was replaced by  $\text{Fe}^{3+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$ , or  $\text{Ni}^{2+}$  (data not shown).

**Purification of the 29-kDa PLC.** The purification procedure was used on 9-h cultures made on charcoal-treated TGY

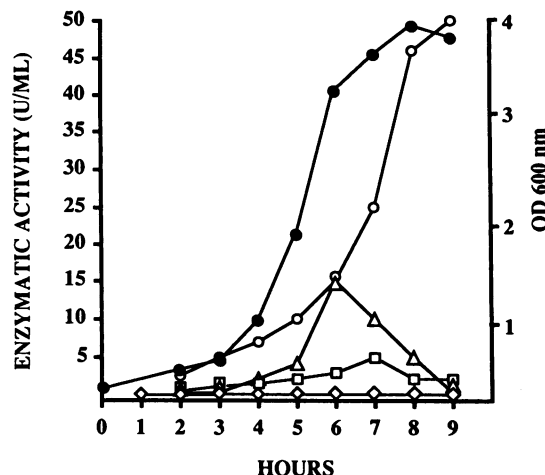


FIG. 1. Kinetics of lecithinase activity in supernatants according to culture conditions. Bacterial growth curves (●) were identical. Lecithinase activities: TGY broth (◇), TGY broth plus  $\text{ZnSO}_4$  (0.1 mM) (□), charcoal-treated TGY broth (Δ), charcoal-treated TGY broth plus  $\text{ZnSO}_4$  (0.1 mM) (○). Bacterial growth was monitored by optical density (OD) at 600 nm, and no difference was observed under the various culture conditions. Enzymatic activity was maximum when bacteria were incubated for 9 h at 37°C in charcoal-treated TGY broth supplemented with  $\text{ZnSO}_4$ .

broth supplemented with  $\text{ZnSO}_4$ . The enzyme was purified from the crude extracellular concentrated supernatant by a three-step procedure. Fractions obtained were checked for phospholipase activity (turbidimetric method) and by SDS-PAGE (Fig. 2). The concentrated supernatants were dialyzed against 0.05 M Tris hydrochloride buffer (pH 7.45) (Fig. 2, lane 1) and applied onto a DEAE-52 cellulose column equilibrated with this buffer at a flow rate of 0.7 ml/min. The unretained material containing about 90% of the initial phospholipase activity was concentrated by ultrafiltration in a stirred cell equipped with a PM10 membrane (Fig. 2, lane 2), and applied onto a Bio-Gel P100 column and eluted with PBS (pH 6.8). The fractions containing enzymatic activity (Fig. 2, lane 3) were pooled, concentrated, and applied onto

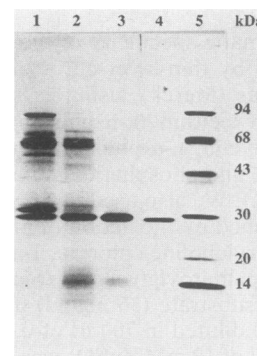


FIG. 2. SDS-PAGE (Coomassie blue staining) of proteins associated with lecithinase activity during the three-step purification procedure of phospholipase of *L. monocytogenes* (see Materials and Methods). Lanes: 1, concentrated supernatant; 2, after DEAE-52 column (step 1); 3, after Bio-Gel P100 column (step 2); 4, after Bio-Gel P100 column (step 3); 5, markers (5 to 10 µg of protein per well). The purified exoenzyme is a 29-kDa protein.

TABLE 1. Purification of an extracellular PLC from *L. monocytogenes*

Purification step	Vol (ml)	Protein (μg/ml)	Activity (U/ml)	Sp act (U/mg of protein)	Recovery (%)
1. Culture supernatant	2,000	75	50	666	
2. Concentrated supernatant	250	175	355	2,028	88
3. DEAE-52	10	650	6,500	10,000	65
4. Bio-Gel P100	4	180	3,500	19,400	14
5. Bio-Gel P100	6	75	2,000	26,000	12

a second Bio-Gel P100 column, which allowed to recover the purified enzyme (26,000 U/mg of protein), as evidenced by the single band observed by SDS-PAGE (Fig. 2, lane 4). A summary of the purification steps, all carried out at 4°C, is presented in Table 1. The pure enzyme appears as a single polypeptide chain of  $M_r$  29,000, visualized as one sharp band on SDS-PAGE after staining with Coomassie blue. Increasing the load of pure toxin up to 5 μg of protein did not reveal any additional band after silver staining (not shown). The isoelectric point of the pure enzyme was determined to be 8.4.

**The 29-kDa enzyme expresses a zinc-dependent lecithinase and sphingomyelinase activities.** The enzymatic activity of the 29-kDa protein was studied by TLC (Fig. 3). After incubation of lecithin or sphingomyelin for 1 and 4 h at 37°C with 10 U of pure enzyme, the migration patterns were analyzed after iodine staining. Lecithin and sphingomyelin were hydrolyzed after 1 h of incubation by the enzyme in the presence of 1 mM ZnSO<sub>4</sub> (Fig. 3, lanes 2 and 6), whereas

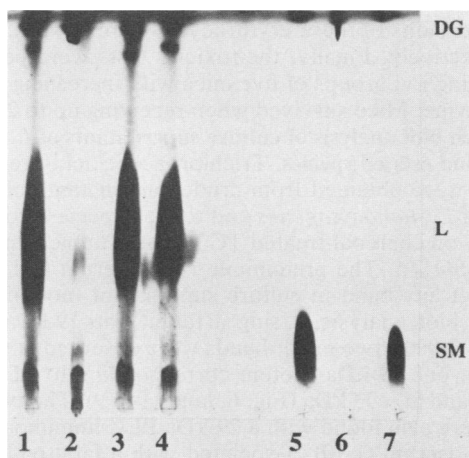


FIG. 3. Zinc-dependent enzymatic activity of the 29-kDa protein from *L. monocytogenes*. Lecithin or sphingomyelin was incubated for 4 h at 37°C with 10 U of pure 29-kDa exoenzyme in the presence of 1 mM ZnSO<sub>4</sub> (see Materials and Methods). Ascending chromatography of lipids extracted with chloroform-methanol (9:1, vol/vol) was carried out on silica gel plates for 2 h in chloroform-methanol-water (65:25:4, vol/vol/vol). Lipids were stained by iodine vapor. Lanes: 1, lecithin; 2, lecithin plus enzyme; 3, lecithin plus enzyme plus 1 mM EDTA; 4, lecithin plus enzyme plus 2 mM EDTA; 5, sphingomyelin; 6, sphingomyelin plus enzyme; 7, sphingomyelin plus enzyme plus 1 mM EDTA. It can be seen that lecithin and sphingomyelin are totally hydrolyzed by the 29-kDa protein in the presence of ZnSO<sub>4</sub>. Enzymatic activity is abrogated by addition of EDTA. L, lecithin; SM, sphingomyelin; DG, diacylglycerol.

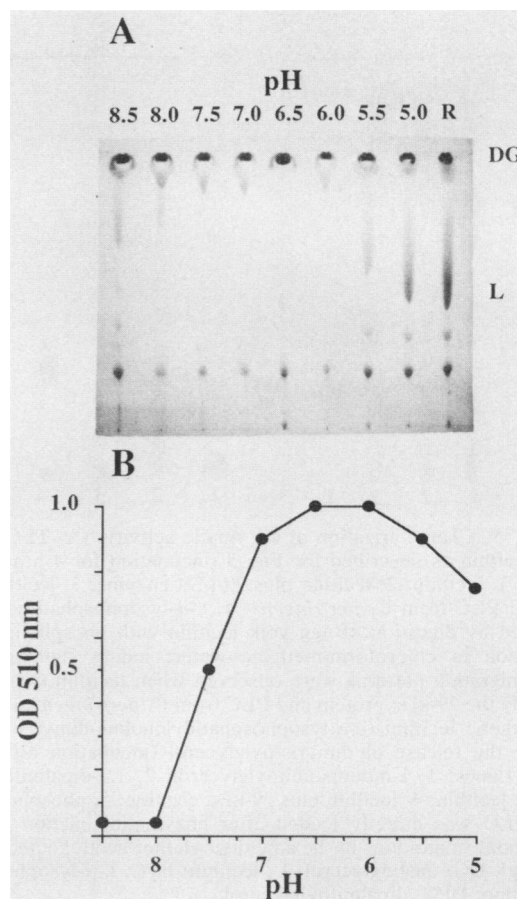


FIG. 4. Effect of pH on enzymatic activity. (A) Lecithin was incubated for 1 h at 37°C with 10 U of 29-kDa enzyme (see Materials and Methods), and lipids were subjected to ascending chromatography as for Fig. 3. Lane R, reference (lecithin alone). DG, diacylglycerol; L, lecithin. (B) Phosphatidylcholine-cholesterol liposomes were incubated for 4 h at 37°C with 200 U of 29-kDa enzyme per ml, and optical density (OD) at 510 nm was measured. The optimum pH of enzymatic activity was between 6 and 7.

sphingomyelin required 4 h for complete hydrolysis. The enzymatic activity was abrogated by addition of 1 to 2 mM EDTA (Fig. 3, lanes 3, 4, and 7), indicating that the lipolytic activity is zinc dependent. The pH range of lecithinase activity was evaluated by incubating for 1 h at 37°C the purified enzyme (10 U) with lecithin at various pHs between 5.0 and 8.5. The released phospholipids were then analyzed by TLC (Fig. 4A). The optimum pH of phospholipase activity was between pH 6 and 7. Similar results were obtained on phosphatidylcholine-cholesterol liposomes treated with the enzyme (Fig. 4B).

**Characterization of the enzymatic activity.** The enzymatic activity of the 29-kDa protein was further characterized by TLC. It is known that lecithin is hydrolyzed by PLC into diacylglycerol and phosphorylcholine and that sphingomyelin is hydrolyzed by sphingomyelinase into ceramide and phosphorylcholine. We first demonstrate that the migration hydrolysis profile of egg yolk lecithin obtained with the 29-kDa protein of *L. monocytogenes* was similar to that observed with pure phospholipase C from *C. perfringens*, giving rise to diacylglycerol (Fig. 5A, lanes 2 and 3), as shown by iodine staining. In contrast, the lecithin hydroly-



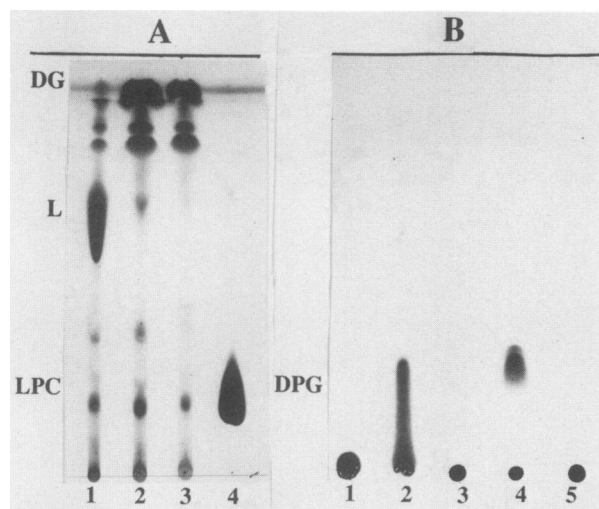


FIG. 5. Characterization of enzymatic activity. (A) TLC of egg yolk lecithin as described for Fig. 3 (incubation for 4 h at 37°C). Lanes: 1, lecithin; 2, lecithin plus 29-kDa enzyme; 3, lecithin plus purified PLC from *C. perfringens*; 4, L- $\alpha$ -lysophosphatidylcholine obtained by digestion of egg yolk lecithin with phospholipase A<sub>2</sub> (migration in chloroform-methanol-water; iodine staining). The same migration patterns were observed when lecithin was hydrolyzed by the 29-kDa protein and PLC from *C. perfringens*. (B) TLC of synthetic lecithin (L- $\alpha$ -lysophosphatidylcholine-dimyristoyl) revealing the release of dimyristoylglycerol (incubation for 4 h at 37°C). Lanes: 1, 1-monopalmitoylglycerol; 2, 1,2-dipalmitoylglycerol; 3, lecithin; 4, lecithin plus 29-kDa enzyme; 5, phosphorylcholine (TLC was directly loaded after enzymatic reaction without extraction; migration in hexane-diethylether-acetic acid; iodine staining). DG, diacylglycerol; L, lecithin; LPC, L- $\alpha$ -lysophosphatidylcholine; DPG, dipalmitoylglycerol.

sate obtained with phospholipase A<sub>2</sub> displayed a quite different pattern, producing L- $\alpha$ -lysophosphatidylcholine (Fig. 5A, lane 4). To distinguish release of diacylglycerol from release of monoacylglycerol during the enzymatic degradation of lecithin, TLC migration was analyzed with hexane-diethylether-acetic acid, using synthetic lecithin (L- $\alpha$ -phosphatidylcholine dimyristoyl) as a substrate, and various controls (1-monopalmitoylglycerol, 1,2-dipalmitoylglycerol, and phosphorylcholine). Lecithin was hydrolyzed into dipalmitoylglycerol (Fig. 5B, lane 4), thus demonstrating the existence of PLC activity. Phosphorylcholine was revealed on this former TLC by molybdate staining (not shown), showing a spot at the origin of lane 5 (phosphorylcholine) and of lanes 3 and 4 (synthetic lecithin plus enzyme [lane 3] and synthetic lecithin [lane 4]). This result (Fig. 5B, lane 4) suggests that nonmigrating phosphorylcholine is released from synthetic lecithin after enzymatic hydrolysis. The 29-kDa enzyme was also capable of hydrolyzing rapidly (1 h at 37°C) purified substrates, such as phosphatidylethanolamine, and phosphatidylserine, but not phosphatidylinositol (not shown). These results indicate that the 29-kDa enzyme is a PLC with a wide substrate specificity but is not phosphatidylinositol phospholipase C (PI-PLC).

**Hemolytic activity and toxicity of the enzyme.** The enzyme displayed a weak, hemolytic activity against erythrocytes which was not altered by addition of 1 mM CaCl<sub>2</sub>. Full expression of the hemolytic activity required 18 h of incubation at 37°C. Detectable lysis was observed with guinea pig, horse, and human erythrocytes but not with sheep erythrocytes. The amounts of toxin required to lyse 50  $\mu$ l of

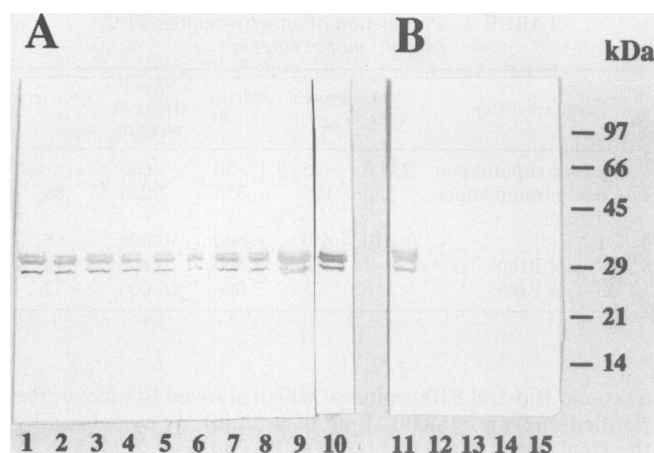


FIG. 6. Western blot analysis of culture supernatants of strains of *L. monocytogenes* and related species. Trichloroacetic acid-precipitated proteins obtained from culture supernatants of charcoal-treated TGY supplemented with 0.1 mM ZnSO<sub>4</sub> were migrated by SDS-PAGE. Electrotransferred proteins were probed with a rabbit anti-29-kDa protein serum (dilution, 1:2,000) except in lane 10, for which an immunoadsorbed anti-29-kDa protein serum was used (dilution, 1/10). (A) *L. monocytogenes*. Lanes: 1, CIP82110<sup>T</sup>; 2, SLCC5132; 3, SLCC3551; 4, SLCC5156; 5, N2661; 6, N74217; 7, N8018; 8, N62262; 9, EGD; 10, EGD. (B) *L. monocytogenes* and related species. Lanes: 11, *L. monocytogenes* EGD; 12, *L. innocua* CIP8011<sup>T</sup>; 13, *L. welshimeri* SLCC5334<sup>T</sup>; 14, *L. seeligeri* CIP100100<sup>T</sup>; 15, *L. ivanovii* CIP 4141<sup>T</sup>. All strains of *L. monocytogenes* produced the 29-kDa enzyme as well as a 33-kDa antigenically related protein that was also recognized by the immunoadsorbed antiserum. Related *Listeria* species do not produce extracellular proteins antigenically related to the 29-kDa PLC from *L. monocytogenes* proteins.

2% suspensions of these erythrocytes were 70, 280, and 200 ng, respectively. Finally, the toxicity tests were performed by injecting i.v. groups of five mice with increasing doses of pure enzyme. Mice survived when receiving up to 25  $\mu$ g i.v.

**Western blot analysis of culture supernatants of *L. monocytogenes* and related species.** Trichloroacetic acid-precipitated proteins were obtained from crude supernatants of various strains of *L. monocytogenes* and related species grown for 9 h at 37°C on charcoal-treated TGY broth supplemented with 0.1 mM SO<sub>4</sub>Zn. The preimmune rabbit serum (1:1,000) did not detect any band in culture supernatant (not shown) by Western blot analysis. Using a rabbit anti-29-kDa protein serum (1:2,000), two major bands were observed at the same intensity, one 29-kDa protein corresponding to PLC and a double band at ~33 kDa (Fig. 6, lanes 1 to 9). The two major bands were also found with a 29-kDa PLC immunoadsorbed rabbit antiserum (1:10), associated with a faint 66-kDa protein (Fig. 6, lane 10), thus indicating that the 33-kDa double band is closely antigenically related to the 29-kDa protein.

With respect to the other *Listeria* species, no bands were detected in culture supernatants of *L. ivanovii*, a species that produced an opalescent zone around colonies on egg yolk agar, or with supernatants from *L. innocua*, *L. seeligeri*, and *L. welshimeri* (Fig. 6B). These results indicate that the 29-kDa PLC is specifically produced by *L. monocytogenes* and not by related species.

**Amino acid composition and partial peptide sequence of the enzyme.** We repeatedly failed to determine the NH<sub>2</sub>-terminal sequence of the protein, which was probably blocked. The 29-kDa protein was then electrotransferred onto Immobilon

and hydrolyzed by trypsin. Peptides were further purified by HPLC. The sequence of one homogeneous peptide, corresponding to an internal part of the molecule, was determined to be Tyr-Phe-Asn-Gln-Ser-Val-Thr-Asp-Tyr.

## DISCUSSION

In this work, a 29-kDa PLC was purified to homogeneity from the supernatant of *L. monocytogenes* EGD. This exoenzyme expresses lecithinase activity and weaker sphingomyelinase activity (Fig. 3), as does the PLC of *C. perfringens* (19, 37). The exoenzyme hydrolyzes phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine but not phosphatidylinositol, thus displaying a wide substrate specificity. These results clearly distinguish the 29-kDa PLC from the PI-PLC recently described in *L. monocytogenes* (22, 26). This PI-PLC is encoded by a gene located upstream from *hlyA* and is strongly homologous to the PI-PLC produced by *Bacillus thuringiensis* and *Bacillus cereus* (22, 26). We found that the 29-kDa enzyme is inactive on phosphatidylinositol and differs in molecular weight from the 36-kDa PI-PLC produced by *L. monocytogenes* (22, 26).

The biological properties of the 29-kDa PLC were further investigated. The exoenzyme was active at a wide pH range (6.0 to 7.0), as shown by TLC and liposome hydrolysis at various pHs (Fig. 4). It was weakly hemolytic for erythrocytes from various animal species, including guinea pig, horse, and human, but was not hemolytic for sheep erythrocytes. Interestingly, sheep erythrocytes are practically devoid of phosphatidylcholine, whereas guinea pig, horse, and human erythrocytes contain 29 to 42% of this compound (33). Moreover, the exoenzyme was not toxic in mice injected i.v. with 25 µg of pure protein, in contrast to, for example, *C. perfringens* PLC (25, 41).

The enzymatic activity was strongly enhanced in culture supernatants when bacteria were grown in charcoal-treated TGY broth supplemented with ZnSO<sub>4</sub> but was not stimulated in the presence of Fe<sup>3+</sup>, Cu<sup>2+</sup>, Co<sup>2+</sup>, or Ni<sup>2+</sup>. These data suggest that the 29-kDa PLC produced by *L. monocytogenes* is zinc dependent. Zinc may act directly on the 29-kDa exoenzyme, as shown on Fig. 3. Interestingly, zinc also plays a critical role in the enzymatic activities of PLCs from *C. perfringens* (19, 31) and *B. cereus* (23), which are metalloenzymes containing two zinc ions essential to their catalytic activity. The 29-kDa PLC from *L. monocytogenes* may also be a metalloenzyme, as is the zinc-dependent metalloprotease recently described in this pathogen (6, 27).

We found that the 29-kDa PLC was produced by all virulent strains of *L. monocytogenes* tested, as shown by Western blot analysis using a rabbit polyclonal immune serum raised against the pure 29-kDa protein. An antigenically related double band at ~33 kDa, as well as a minor 66-kDa band, was also detected in *L. monocytogenes* supernatants. We cannot eliminate the possibility that these additional bands are due to contamination of rabbit antiserum. However, the 33-kDa band is still recognized when antiserum is highly diluted (1:2,000 to 1:4,000), whereas the other bands disappear. The 29- to 33-kDa proteins are also detected by a 29-kDa PLC immunoadsorbed antiserum diluted 1:10, suggesting that the 33-kDa protein is antigenically closely related to PLC. That the 33-kDa protein is an inactive precursor of the 29-kDa PLC remains to be established.

The anti-29-kDa PLC serum did not detect any signal in *L. ivanovii* supernatant, which also produces an opalescent zone on egg yolk agar. It has been demonstrated that *L.*

*ivanovii* produces a 24- to 27-kDa sphingomyelinase (18, 42) devoid of lecithinase activity (42). On the basis of the enzymatic activity, molecular weight, and absence of antigenic cross-reactivity, the 29-kDa PLC of *L. monocytogenes* appears to be different from this sphingomyelinase. With regard to the other species (*L. innocua*, *L. seeligeri*, and *L. welshimeri*) which do not produce the opalescent zone of egg yolk agar, the anti-29-kDa PLC also failed to detect any signal in culture supernatants of these bacteria. Moreover, no cross-reactivity has been detected by Western blot analysis between the 29-kDa PLC and PLCs from *C. perfringens* and *B. cereus* (10). The partial peptide sequence of an internal fragment of the 29-kDa PLC was obtained, revealing the nonapeptide Tyr-Phe-Asn-Gln-Ser-Val-Thr-Asp-Tyr. This nonapeptide was found into the structural gene encoding the 29-kDa PLC which has been recently located and sequenced (41a).

In conclusion, *L. monocytogenes* appears to produce at least two extracellular PLCs, a 29-kDa PLC characterized in this work and a 36-kDa PI-PLC. It has recently been found that a 32-kDa protein expresses a phospholipase activity, as shown on SDS-PAGE overlaid with egg yolk agar (17). Whether this enzyme is different from the 29-kDa PLC described here remains unknown. Interestingly, it has been shown that there exists a common genetic control between listeriolysin O and other determinants, including lipolytic phenotype on egg yolk agar (17). These data suggest that the 29-kDa PLC may be coregulated with other virulence factors, but the role of this enzyme in virulence remains unknown.

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